Reactive Oxygen Species in the Elongation Zone of Maize Leaves Are Necessary for Leaf Extension[1]

Andrés A. Rodríguez, Karina A. Grunberg, and Edith L. Taleisnik*

Instituto de Fitopatologia y Fisiologia Vegetal-Instituto Nacional de Tecnología Agropecuaria, Camino a 60 Cuadras Km 5 1/2, 5119 Córdoba, Argentina

The production and role of reactive oxygen species (ROS) in the expanding zone of maize (*Zea mays*) leaf blades were investigated. ROS release along the leaf blade was evaluated by embedding intact seedlings in 2′,7′-dichlorofluorescein-containing agar and examining the distribution of 2′,7′-dichlorofluorescein fluorescence along leaf 4, which was exposed by removing the outer leaves before embedding the seedling. Fluorescence was high in the expanding region, becoming practically non-detectable beyond 65 mm from the ligule, indicating high ROS production in the expansion zone. Segments obtained from the elongation zone of leaf 4 were used to assess the role of ROS in leaf elongation. The distribution of cerium perhydroxide deposits in electron micrographs indicated hydrogen peroxide (H_2O_2) presence in the apoplast. 2′,7′-Dichlorofluorescein fluorescence and apoplastic H_2O_2 accumulation were inhibited with diphenyleneiodonium (DPI), which also inhibited $O^{\bullet}{}_2^-$ generation, suggesting a flavin-containing enzyme activity such as NADPH oxidase was involved in ROS production. Segments from the elongation zone incubated in water grew 8% in 2 h. KI treatments, which scavenged H_2O_2 but did not inhibit $O^{\bullet}{}_2^-$ production, did not modify growth. DPI significantly inhibited segment elongation, and the addition of H_2O_2 (50 or 500 μM) to the incubation medium partially reverted the inhibition caused by DPI. These results indicate that a certain concentration of H_2O_2 is necessary for leaf elongation, but it could not be distinguished whether H_2O_2, or other ROS, are the actual active agents.

In monocots, leaf growth is restricted to the leaf base where cell division and expansion occur (Langer, 1979). Cell growth is controlled by water uptake and the rheological properties of the cell walls (Ray, 1987), and cell expansion requires first the loosening of cell walls, which thus can yield to the pressure exerted by the symplasm. Expansion ceases as cells become less extensible by incorporating cross-linking phenolic compounds and an insoluble Thr-rich protein (Carpita and McCann, 2000).

Cell wall loosening has been regarded as a process mainly catalyzed by expansins (McQueen-Mason, 1995), hydrolases such as endoglucanases, and xyloglucan endotransglycosilase (Cosgrove, 1999). Recently, in vitro studies have shown that nonenzymatic processes involving reactive oxygen species (ROS) cause wall polysaccharide scission (Miller, 1986; Fry, 1998; Schweikert et al., 2000). For this process to occur in vivo, ROS must be present in the apoplast, and apoplastic ROS accumulation has been shown many plant tissues (Schopfer, 1994). Apoplastic ROS generation is believed to be associated to several mechanisms that include participation of peroxidases (Chen and Schopfer, 1999), ascorbate (Fry, 1998), and the activity of plasmalemma NAD(P)H oxidase (Ogawa et al., 1997), similar to the one found in mammalian phagocytes (Babior et al., 1997; Lamb and Dixon, 1997). This enzyme catalyzes the reduction of O_2 to $O^{\bullet}{}_2^-$, which can then originate various other ROS by enzymatic and nonenzymatic processes (Asada, 1994). Hydrogen peroxide (H_2O_2) appears to be the main and more stable product of O_2 reduction, resulting from nonenzymic processes operating at physiological pH in the cell wall, and from the activity of apoplastic superoxide dismutase (SOD; Ogawa et al., 1997; Schopfer et al., 2001). Expanding organs such as embryonic axes (Puntarulo et al., 1988), growing roots (Jon et al., 2001), and germinating seeds (Schopfer et al., 2001) can extrude ROS, which have been suggested to be involved in plant defense and in signaling. The presence of H_2O_2 was shown to be necessary for tobacco (*Nicotiana tabacum*) protoplast division (de Marco and Roubelakis-Angelakis, 1996). In ripening pear (*Pyrus communis*) fruit cell walls, evidence for ·OH radical attack on wall polysaccharides supported the hypothesis that ROS participate in wall softening during maturation (Fry et al., 2001). Auxin-induced elongation growth could be inhibited by ·OH scavengers (Schopfer, 2001). The purpose of this work is to assess the role of ROS in elongation growth in the expanding zone of maize (*Zea mays*) leaf blades.

RESULTS AND DISCUSSION

In Vivo Detection of ROS in a Growing Maize Leaf

The first step in this work was to characterize growth and ROS extrusion in an actively growing

[1] This work was supported by the Agencia de Promoción Científica y Tecnológica of Argentina (Fondo para la Investigación Cientifica y Tecnológica grant no. 6869) and by the Fundación Antorchas (grant no. 13740/1–115). This work is part of A.A.R.'s doctorate.

* Corresponding author; e-mail gertale@cordoba.com.ar; fax 0054–351–4974330.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.001222.

maize leaf blade. In blades of leaf 4 that were actively growing, the distribution of segmental elongation rates (SER) within the elongation zone (Fig. 1) had a typical tailed bell shape (Volenec and Nelson, 1981; Bernstein et al., 1993) and SER were maximal between 5 and 15 mm from the ligule. The elongation zone was less than 40 mm long, which is within the range reported for other maize cultivars (de Souza and MacAdam, 2001).

A commonly used reagent to detect ROS is 2′,7′-dichlorofluorescin (DCFH), which is oxidized by ROS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF; Simontacchi et al., 1993; Schopfer et al., 2001). DCFH exhibits selectivity for H_2O_2 over other free radicals (Allan and Fluhr, 1997); nevertheless, the DCFH assay provides an integral measure for several ROS (H_2O_2, ·OH, and $O^{\bullet}_2{}^{-}$) because it is likely that in vivo, other radical species are quickly converted to the more stable H_2O_2 (Abeles, 1986). When ROS-producing tissues are embedded in agar-containing DCFH (Schopfer et al., 2001), a bright-green fluorescence is seen upon illumination with UV light.

The question of whether ROS are produced in the growing zone of the leaf blade was evaluated by embedding intact seedlings in DCFH-containing agar (Fig. 1). Leaf 4 was exposed by removing the outer leaves before embedding the seedling. DCF fluorescence was high in the expanding region, and decreased further up the blade, becoming practically non-detectable beyond 65 mm from the ligule (Fig. 1). These results indicate that ROS production, and extrusion, were high in the expanding region, and almost nil in the expanded one.

To evaluate the participation of ROS in elongation growth, we decided to use excised segments, obtained from the growing region (0–20 mm from the ligule, segments from the elongation zone [SEZ]). Such segments can grow for at least 2 h after excision (Neves-Piestun and Bernstein, 2001) and can be easily subject to ROS-modifying treatments by incubating in appropriate solutions. Isolated coleoptile segments have thus been treated to assess the effects of H_2O_2 on growth and cell wall stiffening (Schopfer, 1996). SEZ reproduced the ROS extrusion pattern observed in intact leaves: Excised segments from the expanding zone were bright green, whereas those from the expanded blade did not fluoresce (Fig. 2). The brighter fluorescence observed along the cut edges of both segments are probably ROS generated by mechanical stress and wounding (Low and Merida, 1996; Orozco-Cárdenas et al., 2001). To rule out permeability differences between the expanding and expanded region, ROS were measured in apoplast fluid. The activity of Glc-6-P dehydrogenase, a cytoplasmic enzyme, was not detectable in apoplastic extracts, indicating they were essentially free from

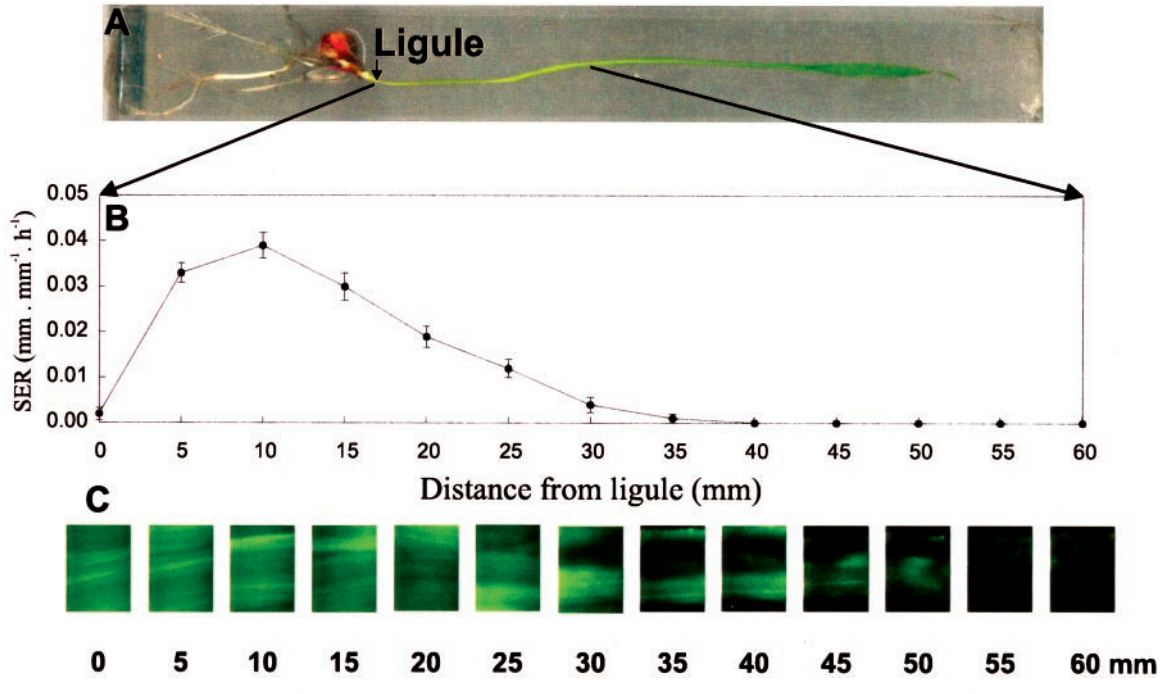

Figure 1. Spatial distribution of elongation growth and ROS production in a maize leaf 4. A, Setup used for determining ROS production along intact leaf blades. Leaf 4 was exposed by removing the outer leaves and the whole seedling was embedded in DCFH-containing agar and examined 30 min later under a microscope with epifluorescence. Actual size. B, Spatial distribution of SER. Each point is the average SER for the 5-mm segment ending at the distances shown in the graph. The first point displaced very little from the ligule. Data are means ± SE of $n = 10$ leaves. C, DCF fluorescence along leaf 4 blade, indicating ROS release. Successive images were taken along the blade. The numbers indicate the distance (mm) from the left border of the segment to the ligule. Magnification: 32×.

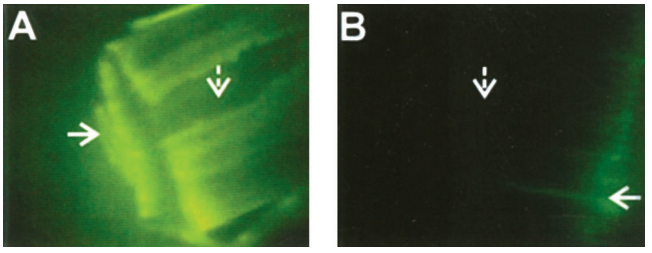

Figure 2. ROS release from cut edges of maize leaf blade segments, 30 min after embedding in DCFH-containing agar. A, Expanding zone. B, Expanded blade. Arrows indicate the cut edge, and dashed arrows indicate the blade beyond the cut. Magnification: 32×.

cytoplasmic contamination (Table I). The percentage decrease in DCF fluorescence due to ascorbate, a nonspecific ROS scavenger, was 34.75 and 7.14 (means for $n = 6$) for apoplastic fluid from the expanding and expanded zones, respectively, indicating ROS concentration was significantly (Student's t test, $P < 0.001$) higher in the expanding zone.

Modulating ROS Concentration

To determine whether ROS are necessary for leaf blade expansion, we subjected segments to treatments with KI and diphenyleneiodonium (DPI), and assessed the effects on ROS presence and growth. KI is a known H_2O_2 scavenger (Frahry and Schopfer, 1998a) and DPI is a suicide inhibitor of the phagocytic NADPH oxidase and also an inhibitor of NADH-dependent H_2O_2 production by peroxidase (Frahry and Schopfer, 1998b) and has been used to reduce ROS production in plant systems (Ros Barceló, 1998; Schopfer et al., 2001).

Observations of electron micrographs showed ROS in the apoplast of the leaf expansion zone (Fig. 3A), as verified by the distribution of cerium perhydroxide deposits (Bestwick et al., 1998). Treatments with catalase also showed a decreased number of crystals, confirming cerium perhydroxides deposition was due to H_2O_2 (Fig. 3B). KI-treated segments showed no cerium perhydroxides deposition (Fig. 3C). KCl was tested as control to rule out effects of salt concentration (10 mM) on H_2O_2 production, and KCl-treated segments (Fig. 3D) looked like controls (Fig. 3A).

DPI-treated segments did not stain for apoplastic H_2O_2 (Fig. 3E), suggesting NAD(P) H contributed to ROS generation in maize SEZ.

DPI- and KI-treated SEZ had quenched fluorescence when compared with non-treated control segments (Fig. 4), and with segments treated with the same concentration of KCl, confirming the results from the electron micrographs.

DPI inhibition of ROS presence in the apoplast suggested a flavin-containing enzyme activity such as NAD(P)H oxidase was involved in ROS generation. Another indication of NAD(P)H oxidase activity was the presence of $O^{\bullet}_2{}^{-}$. NAD(P)H oxidase can generate $O^{\bullet}_2{}^{-}$, which is dismutated to H_2O_2 and O_2 either spontaneously or enzymatically by intervention of SOD (Schopfer et al., 2001). $O^{\bullet}_2{}^{-}$ production can be measured by the oxidation of Na,3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium](4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT). In the presence of $O^{\bullet}_2{}^{-}$, XTT produces a colored formazan that can be measured spectrophotometrically. Formazan production was significantly inhibited in DPI-treated segments (Fig. 5A), providing further support to the idea that apoplastic ROS in SEZ are produced as $O^{\bullet}_2{}^{-}$, presumably by NAD(P)H oxidase intervention. Sodium azide (NaN_3) inhibits apoplastic Cu-Zn SOD and peroxidases (Ogawa et al., 1997), and, thus, would lead to increased $O^{\bullet}_2{}^{-}$ accumulation. Also, as expected, it increased formazan formation (Fig. 5). Because $O^{\bullet}_2{}^{-}$ is known to dismutate spontaneously to H_2O_2, SEZ treated with NaN_3 fluoresced in the presence of DCFH (not shown), as it was expected. These results, taken together, support the idea that NAD(P)H oxidase participates in apoplastic ROS production in growing maize leaves. Treatments with KI or KCl had no effect on $O^{\bullet}_2{}^{-}$ production, and formazan formation in those treatments was similar to the controls.

Effects of ROS on Growth

SEZ incubated in water grew 8% in 2 h (Fig. 6). DPI treatments, which inhibited the production of $O^{\bullet}_2{}^{-}$ and, therefore, any ROS resulting from it, significantly inhibited segment elongation (Fig. 6). The addition of H_2O_2 (50 and 500 μM) to the incubation medium partially reverted the elongation inhibition caused by DPI. Although these results indicate that a certain concentration of H_2O_2 is necessary for leaf elongation, they do not provide information on whether H_2O_2, or other ROS, are the actual active

Table I. *Determination of the contamination of the apoplastic extracts (means ± SE, n = 3)*

Protein Source	Glc-6-P Dehydrogenase Specific Activity	Protein Concentration
	mM NAD(P)H mg^{-1} protein min^{-1}	*mg mL^{-1}*
Apoplastic extract		
Expanding zone	−0.003 ± 0.000	0.589 ± 0.013
Expanded zone	−0.004 ± 0.002	0.188 ± 0.002
Whole-tissue homogenate		
Expanding zone	0.017 ± 0.001	3.616 ± 0.082
Expanded zone	0.035 ± 0.003	3.134 ± 0.299

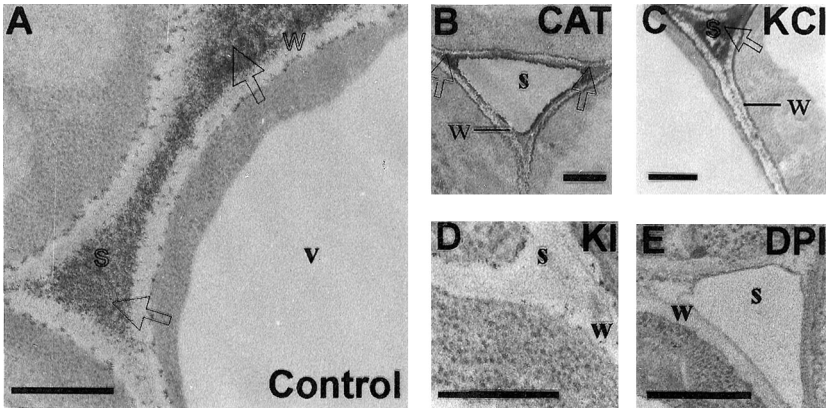

Figure 3. Cellular cytochemical localization of H_2O_2 in mesophyll cells in the expansion zone of maize leaf blades. Electron-dense deposits of cerium perhydroxide (transparent arrows) are seen in the apoplast. Segments had been incubated for 2 h in water (A), 100 units mL^{-1} catalase (B), 10 mM KCl (C), 10 mM KI (D), or 200 μM DPI (E). w, Cell wall; s, intercellular space; v, vacuole. Black bars represent 0.5 μm.

agents. The oxidative scission of plant cell wall polysaccharides could also be achieved by other radicals, generated in the cell wall in the presence of H_2O_2. Ascorbate-induced ·OH radicals were proposed to cleave wall polysaccharides in vitro (Fry, 1998; Schweikert et al., 2000). This mechanism is thought to operate in vivo as well because ·OH can be generated in the presence of H_2O_2, O_2, ascorbate, and Cu^{2+} (Halliwell and Gutteridge, 1990), which are all usually present in the apoplast.

KI treatments scavenged H_2O_2 (Fig. 3) but did not modify growth (Fig. 6). These results could be interpreted to indicate that H_2O_2 had no effect on elongation; however, Schopfer et al. (2001) reported only a 47% decrease in H_2O_2 release by 10 mM KI acting on radish (*Raphanus sativus*) seed coats. Though we could not observe cerium perhydroxide crystals in the KI treatment, it is possible that transient presence of small concentrations of H_2O_2, would not be detected by $CeCl_3$. Because ROS generation was maintained in the KI treatment (Fig. 5), this provides support to the idea that while (an) other ROS may be the primary agent required for growth promotion, the lower exogenous H_2O_2 concentrations supplied (50 or 500 μM) may be sufficient for sustaining its generation in the absence of $O^{\cdot}{}_2^-$ production (DPI treatment).

Growth inhibition by DPI could not be reverted by higher H_2O_2 concentrations (5 mM). This was not unexpected, given the proposed role of H_2O_2 in wall stiffening (Schopfer, 1996).

It has been suggested that the action of ROS in the apoplast may involve a delicate balance between cleavage and cross-linking activities (Cosgrove, 1999). This may be associated to a differential activity

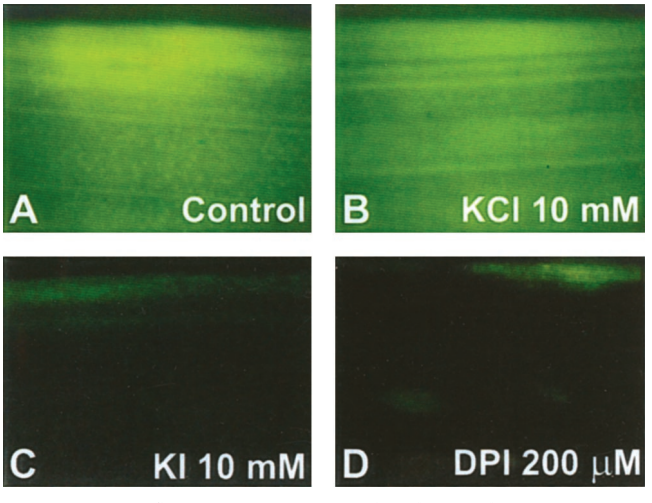

Figure 4. DCF fluorescence in KI- and KCl-treated SEZ. SEZ were incubated for 2 h in aerated media and then embedded in the same medium containing 1% (w/v) agar and 10 μM DCFH. Fluorescence images were taken after 30 min. A, Water only (control); B, 10 mM KCl; C, 10 mM KI; D, 200 μM DPI. Magnification: 32×.

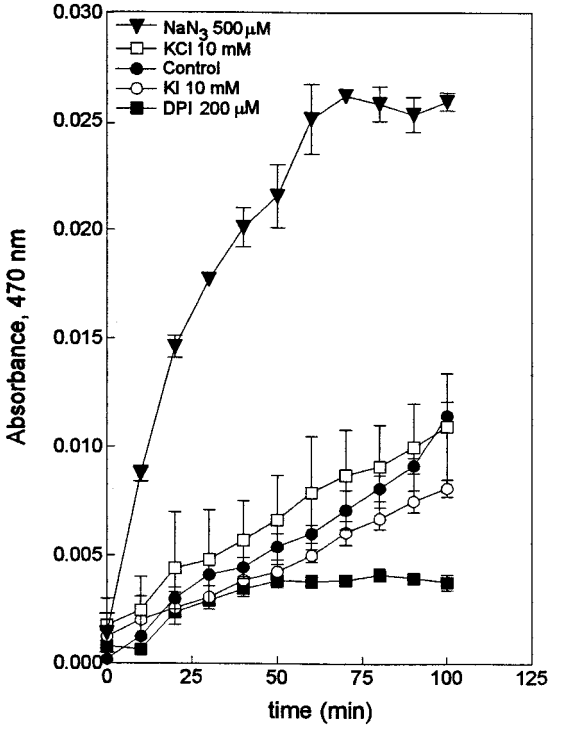

Figure 5. Kinetics of formazan production from XTT in SEZ incubated in water (control), 200 μM DPI, 500 μM NaN_3, 10 mM KI, or 10 mM KCl. Results are means ± SE of two independent repetitions of the experiment, using 10 segments each time.

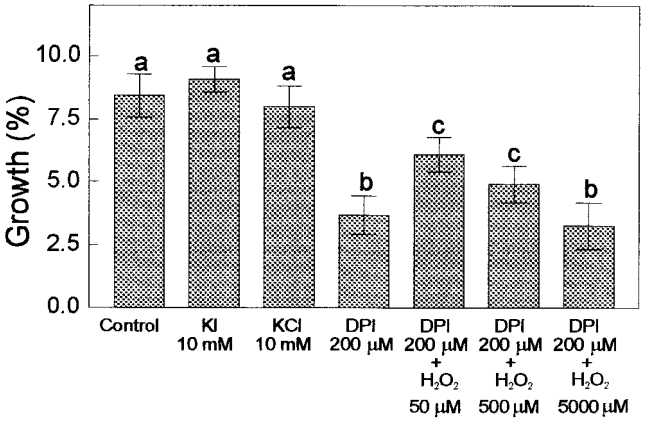

Figure 6. Effect of treatments that modify apoplastic ROS concentration on the growth of maize leaf segments. SEZ were incubated in aerated media: water (control), 10 mM KI, 10 mM KCl, 200 μM DPI, or 200 μM DPI with various concentrations of H_2O_2. Results are percentage length increase during a 2-h incubation period, and are means ± SE of 10 segments each. Columns with different letters are significantly different ($P < 0.05$).

of cell wall peroxidases because different soluble peroxidase isozymes characterize the expanding and expanded region in maize leaves (de Souza and MacAdam, 2001) and in *Festuca arundinacea* (MacAdam et al., 1992), and were suggested to have different roles in cell wall growth. ROS action on growth could conceivably be exerted through a promotion of cell wall polysaccharide cleavage in vivo (Schopfer, 2001), such as operates in vitro (Miller, 1986; Fry, 1998; Schweikert et al., 2000), and this possibility is currently being explored. H_2O_2 could also be acting as a signal molecule. H_2O_2 readily crosses membranes, and is known to be an activator of some MAP kinases cascades and can also regulate the expression of certain genes (Bowler and Fluhr, 2000). Both actions could contribute to the observed results.

MATERIALS AND METHODS

Plant Material

Seeds of maize (*Zea mays* cv Prozea 30, Produsem, Pergamino, Argentina) were sown on moist vermiculite contained in plastic net frames placed over a 4.5-L black plastic tray containing water. Trays were kept at 25°C under a light panel of fluorescent and incandescent lights providing 95 μmol photons m^{-2} s^{-1} illumination, with a 12-h photoperiod. When the second leaf emerged, the water was changed to one-half-strength Hoagland solution (Hoagland and Arnon, 1950).

Growth Measurements

SER within the blade expansion zone was calculated from the displacement of pinpricks in a 24-h interval according to Schnyder et al. (1987). A pricking device was made with a series of fine needles spaced 5 mm apart and mounted between two pieces of plexiglas. The basal zone of the seedling was pricked through the sheath whorl and, after 24 h, the sheath whorl was opened and the fourth leaf exposed and examined under a stereoscopic microscope to determine the distance between marks.

Elongation in isolated segments from leaf 4 was measured according to Neves-Piestun and Bernstein (2001). Segments comprising the first 20 mm from the leaf base (ligule), which contained the most actively growing zone (SEZ), were gently vacuum infiltrated and incubated for 2 h in aerated water or in various treatment solutions. Digital images of each segment were obtained before and after the incubation period, by means of a scanner (AGFA Snapscan Touch, Agfa-Gevaert Group, Morstel, Belgium), and length measurements were obtained with an image processing software (Optimas 6.1, Optimas Corporation, Bothell, WA). Growth was expressed as percentage length increase in that period.

Measurement of ROS Release

In vivo determination of ROS release along the fourth leaf or in leaf segments was performed by a modification of the agar technique described by Schopfer et al. (2001). The fourth leaf was exposed by removing the outer leaves. DCFH-containing agar was prepared by adding an appropriate volume of a 25 mM DCFH-diacetate (DA) ethanol solution to a 1% (w/v) agar solution in 20 mM phosphate buffer pH 6, to obtain a 50 μM DCFH-DA mixture. The mixture was heated to solubilize the agar; then, leaf segments or entire shoots (attached to the seeds and roots) were embedded when the temperature was close to 30°C as the agar cooled down. Epifluorescence was observed after 30 min with a microscope (Axiophot, Zeiss, Jena, Germany) with excitation filter BP 450–490 and emission filter LP 520. Images were taken with a video camera (SONY DXC-950P, Sony, Tokyo).

To quantify ROS in the apoplast fluid, segments, obtained from the expanded and expanding regions of leaf 4 were isolated and washed for 5 to 7 min to wash ROS released as a consequence of the incision. Segments were then placed, three per tube, in 5 mL of water and subjected to gentle infiltration for 1 min to release trapped air. Segments were then carefully introduced into perforated tubes placed in a non-perforated one (Eppendorf Brinkmann Instruments, Westbury, NY). The tubes were first given a centrifuge pulse at low speed to drain them and were then centrifuged at 2,000*g* for 1 min. Approximately 10 μL of fluid was obtained per group of three segments. The exudate was diluted in 5 mL of water, and each sample was divided into two. Ascorbate (10 mM), a nonspecific ROS scavenger, was added to one of the subsamples, allowed to react for 15 min, and then DCFH-DA was added to both parts to a final concentration of 25 μM, incubated at 33°C for 30 min, and fluorescence was read in a spectrofluorometer (Shimadzu RF-1501, Shimadzu, Kyoto) with excitation/emission wavelengths set to 485 and 525 nm, respectively. For each sample, specific absorbance due to ROS was calculated as percentage fluorescence decrease by ascorbate. Cytoplasmic contamination of the apoplast fluid was verified (in parallel samples) by determining Glc-6-P dehydrogenase activity essentially as described by Kornberg and Horecker (1955). Protein concentration was determined according to Bradford (1976).

H_2O_2 Localization

Cellular H_2O_2 localization was determined cytochemically from cerium perhydroxide deposition after reaction of $CeCl_3$ with endogenous H_2O_2 as described by Bestwick et al. (1997). Positive staining was detected in electron micrographs as the formation of electron-dense deposits. Three-millimeter leaf segments were rinsed in water, infiltrated in various treatment solutions for 1 h (see figure legends), and then transferred to a 5 mM $CeCl_3$ solution in 5 mM MOPS, pH 7.2, and incubated for 3 h. Segments were then fixed in glutaraldehyde and processed for electron microscopy.

Determination of $O^{\cdot}{}_2^-$ Production

The reduction of XTT, which produces a soluble formazan, was used to measure $O^{\cdot}{}_2^-$ production (Frahry and Schopfer, 2001). Groups of 10 segments from the leaf expansion zone were gently infiltrated and incubated in 3 mL of aqueous solution containing 500 μM XTT plus one of the following: 10 mM KI, 10 mM KCl, or 500 μM NaN_3. The effect of DPI treatments on $O^{\cdot}{}_2^-$ production was determined by first infiltrating and incubating the segments in a 200 μM DPI solutions for 2 h, and then transferring them to the XTT solution for formazan detection. Controls were incubated in water plus XTT. Aliquots were obtained at 10-min intervals for 100 min and read at 470 nm in a spectrophotometer (Beckman DU Series 600, Beckman Instruments, Fullerton, CA).

Statistical Analysis

Data were analyzed by appropriate Student's *t* tests or ANOVA, in which case significant differences between individual treatments were determined by Tukey's test or LSD (Complete Statistical Systems, Statsoft, Inc., Tulsa, OK).

ACKNOWLEDGMENTS

The authors are thankful to Dr. Samuel Taleisnik for fruitful discussions. The help of Leandro Ortega and Alicia Córdoba is gratefully acknowledged.

Received December 11, 2001; returned for revision February 20, 2002; accepted April 26, 2002.

LITERATURE CITED

Abeles FB (1986) Plant chemiluminescence. Ann Rev Plant Physiol **37:** 49–72

Allan A, Fluhr R (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. Plant Cell **9:** 1559–1572

Asada K (1994) Production of active oxygen species in photosynthetic tissue. *In* CH Foyer, PM Mollineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC, Boca Raton, FL, pp 77–104

Babior BM, El Benna J, Chanock SJ, Smith RM (1997) The NADPH oxidase of leukocytes: the respiratory burst oxidase. *In* JG Scandalios, ed, Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, Plainview, NY, pp 737–783

Bernstein N, Läuchli A, Silk WK (1993) Kinematics and dynamics of sorghum (*Sorghum bicolor* L.) Leaf development at various Na/Ca salinities: I. Elongation growth. Plant Physiol **103:** 1107–1114

Bestwick CS, Brown IR, Bennet MHR, Mansfield JW (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv phaseolicola. Plant Cell **9:** 209–221

Bestwick CS, Brown IR, Mansfield JW (1998) Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol **118:** 1067–1078

Bowler C, Fluhr R (2000) The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant Sci **5:** 241–246

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Carpita N, McCann M (2000) The cell wall. *In* BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 52–108

Chen SX, Schopfer P (1999) Hydroxyl-radical production in physiological reactions: a novel function of peroxidase. Eur J Biochem **260:** 726–735

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. Annu Rev Plant Physiol Mol Plant Biol **50:** 391–417

de Marco A, Roubelakis-Angelakis KA (1996) Hydrogen peroxide plays a bivalent role in the regeneration of protoplasts. J Plant Physiol **149:** 109–114

de Souza IRP, MacAdam JW (2001) Gibberellic acid and dwarfism effects on the growth dynamics of B73 maize (*Zea mays* L.) leaf blades: a transient increase in apoplastic peroxidase activity precedes cessation of cell elongation. J Exp Bot **52:** 1673–1682

Frahry G, Schopfer P (1998a) Hydrogen peroxide production by roots and its stimulation by exogenous NADH. Physiol Plant **103:** 395–404

Frahry G, Schopfer P (1998b) Inhibition of O_2-reducing activity of horseradish peroxidase by diphenyleneiodonium. Phytochemistry **48:** 223–237

Frahry G, Schopfer P (2001) NADH stimulated, cyanide resistant superoxide production in maize coleoptiles analyzed with tetrazolium based assay. Planta **212:** 175–183

Fry SC (1998) Oxidative scission in plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. Biochem J **332:** 507–515

Fry SC, Dumville JC, Miller JG (2001) Fingerprinting of polysaccharides attacked by hydroxyl radicals *in vitro* and in the cell walls of ripening pear fruit. Biochem J **357:** 729–737

Halliwell B, Gutteridge JMC (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *In* L Packer, AN Glazer, eds, Methods in Enzymology. Academic Press, New York, pp 1–85

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. California Agric Exp Stn Circ **347:** 1–32

Jon JH, Bae YS, Lee JS (2001) Role of auxin-induced reactive oxygen species in root gravitropism. Plant Physiol **126:** 1055–1060

Kornberg A, Horecker BL (1955) Glucose-6-phosphate dehydrogenase. *In* S Colowick, N Kaplan, eds, Methods in Enzymology, Vol I. Academic Press Inc., Publishers, New York, pp 323–325

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 25–275

Langer RHM (1979) How Grasses Grow. Edward Arnold, London, pp 1–60

Low PS, Merida JR (1996) The oxidative burst in plant defense: function and signal transduction. Physiol Plant **96:** 533–542

MacAdam JW, Sharp RE, Nelson CJ (1992) Peroxidase activity in the leaf elongation zone of tall fescue: II. Spatial distribution of apoplastic bound peroxidase activity in genotypes differing in length of the elongation zone. Plant Physiol **99:** 879–885

McQueen-Mason SJ (1995) Expansins and cell wall expansion. J Exp Bot **46:** 1639–1650

Miller AR (1986) Oxidation of cell wall polysaccharides by hydrogen peroxide: a potential mechanism for cell wall breakdown in plants. Biochem Biophys Res Commun **141:** 238–244

Neves-Piestun BG, Bernstein N (2001) Salinity induced inhibition of leaf elongation in maize is not mediated by changes in cell wall acidification capacity. Plant Physiol **125:** 1419–1428

Ogawa K, Kanematsu S, Asada K (1997) Generation of superoxide anion and localization of Cu Zn-superoxide dismutase in the vascular tissue of spinach hypocotyls: their association with lignification. Plant Cell Physiol **38:** 1118–1126

Orozco-Cárdenas ML, Narváez-Vásquez J, Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell **13:** 179–191

Puntarulo S, Sanchez R, Boveris A (1988) Hydrogen peroxide metabolism in soybean axes at the onset of germination. Plant Physiol **86:** 626–630

Ray PM (1987) Principles of plant cell expansion. *In* DJ Cosgrove, DP Kneivel, eds, Physiology of Cell Expansion During Plant Growth. American Society of Plant Physiologists, Rockville, MD, pp 1–27

Ros Barceló A (1998) The generation of H_2O_2 in the xylem of *Zinnia elegans* is mediated by an NADPH-oxidase-like enzyme. Planta **207:** 207–216

Schnyder H, Nelson C, Coutts JH (1987) Assessment of spatial distribution of growth in the elongation zone of grass leaf blades. Plant Physiol **85:** 290–293

Schopfer P (1994) Histochemical demonstration and localization of H_2O_2 in organs of higher plants by tissue printing on nitrocellulose paper. Plant Physiol **104:** 1269–1275

Schopfer P (1996) Hydrogen peroxide mediated cell wall stiffening in vitro maize coleoptiles. Planta **199:** 43–49

Schopfer P (2001) Hydroxyl radical-induced cell-wall loosening in vitro and in vivo: implications for the control of elongation growth. Plant J **28:** 679–688

Schopfer P, Plachy C, Frahry G (2001) Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiol **125:** 1591–1602

Schweikert C, Liszkay A, Schopfer P (2000) Scission of polysaccharides y peroxidase-generated hydroxyl radicals. Phytochemistry **53:** 565–570

Simontacchi M, Caro A, Fraga CG, Puntarulo S (1993) Oxidative stress affects α-tocopherol content in soybean embryonic axes upon imbibition and germination. Plant Physiol **103:** 949–953

Volenec JJ, Nelson CJ (1981) Cell dynamics in leaf meristems of contrasting tall fescue genotypes. Crop Sci **21:** 381–385